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Enantioselective liver microsomal sulphoxidation of albendazole in cattle: effect of nutritional status

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1. The enantioselective liver microsomal sulphoxidation of the benzimidazole anthelmintic, albendazole (ABZ), by cattle liver microsomes has been investigated. The influence of nutritional condition on this biotransformation process was also characterized.

2. ABZ was oxidized to its sulphoxide metabolite (ABZSO) in a NADPH concentration-dependent reaction and the (+) and (−) ABZSO enantiomers formed were identified.

3. V_{max} (0.27 nmol ABZSO formed per min.mg^{-1} microsomal protein) and K_m (15.10 μM) for ABZ sulphoxidation by cattle liver microsomes were obtained. Different V_{max} (0.11 and 0.16 nmol.min^{-1}.mg^{-1}) and K_m (9.40 and 26.70 μM) characterized the enantioselective formation of (+) and (−) ABZSO antipodes, respectively.

4. Free fatty acid (FFA) concentrations and β-hydroxybutyrate concentrations (β-OHB) in serum and liver homogenates were significantly higher in feed-restricted (poor nutritional condition) compared with control animals in an optimal nutritional status. Serum protein concentrations and liver cytosolic glucose 6-phosphate dehydrogenase (G6PD) activity were significantly lower in the feed-restricted compared with control calf.

5. Animal nutritional condition affected the pattern of ABZ sulphoxidation. A higher K_m for (total) ABZSO and (+) ABZSO production was observed in the calf subjected to a period of undernutrition.

6. A nutritionally induced impairment in the affinity of microsomal mixed-function oxidases responsible of ABZ oxidation may be responsible for the observed changes in the liver microsomal sulphoxidation of ABZ in the feed-restricted calf. Furthermore, undernutrition may affect primarily the FMO-mediated formation of (+) ABZSO. These *in vitro* observations agree with the changes observed *in vivo* following the administration of ABZ to the calf subjected to a dietary restriction.

Introduction

Benzimidazole (BZD) and pro-BZD anthelmintics are extensively metabolized in the host (Gottschall *et al.* 1990). The biotransformation process tends to transform these molecules into more polar and less active metabolites, which facilitates their elimination. This metabolic pattern and the resultant pharmacokinetic behaviour are relevant in the attainment of high and sustained concentrations of pharmacologically active drug/metabolites at the target parasite (Lanusse and Prichard 1993). Albendazole (ABZ), methyl-[(5-propylthio)-1H-benzimidazol-2-yl] carbamate, is a BZD derivative that has been used in our laboratory as a model compound to characterize factors affecting pharmacokinetic and biotransformation processes in ruminants. After oral/intraruminal administration of ABZ to cattle, the parent compound is undetectable in plasma, while albendazole sulphoxide (ABZSO) and sulphone ($ABZSO_2$) are the major metabolites recovered (Prichard *et al.* 1985, Sánchez *et al.* 1996). The absence of ABZ parent drug in plasma has been attributed

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to a first-pass oxidation in the liver. In fact, the sequential oxidation of ABZ into ABZSO and $ABZSO_2$ has been shown to be catalysed by liver microsomal mixed-function oxidases in rat (Fargetton *et al.* 1986), pig (Souhaili El Amri *et al.* 1987), lamb (Galtier *et al.* 1986) and calf (Lanusse *et al.* 1993). Sulphoxidation appears to be a rapid and reversible process that equilibrates with the respective thioether (Gyurik *et al.* 1981), although the equilibrium favours metabolism towards sulphoxidation. A portion of the anthelmintically active sulphoxide metabolite undergoes a second, slower and irreversible oxidative step that forms the inactive sulphone metabolite.

The chiral nature of the sulphoxide derivative has been previously described. Two enantiomers of the ABZSO metabolite were identified in plasma after oral administration of ABZ to cattle (Delatour *et al.* 1991a) and other animal species (Delatour *et al.* 1991a, b, Benoit *et al.* 1992). It has been demonstrated that the (+) ABZSO enantiomer represents 91 % of the total ABZSO plasma area under the concentration versus time curve (AUC) in cattle. Such differences between the plasma availability of (+) and (−) ABZSO were attributed to enantioselective differences in ABZ sulphoxidation.

Several host-related factors, affecting either the plasma disposition kinetics and/or the biotransformation process, may influence the anti-parasite activity of BZD compounds. Starvation before treatment in cattle (Sánchez *et al.* 1997) and a reduction in feed intake level in sheep (Alí and Hennessy 1995) enhanced the dissolution and absorption of BZD molecules by reducing the gastrointestinal (GI) transit time. Parasite-mediated inflammatory reactions induce changes in both absorption and ionic trap-mediated distribution of different BZD metabolites (Marriner *et al.* 1985, Hennessy *et al.* 1993, Alvarez *et al.* 1997). Similarly, parasite-mediated liver damage with reduced enzymatic activity affects biotransformation in the *Fasciola hepatica*-infected rat (Tekwani *et al.* 1988) and sheep (Galtier 1992). Previous results obtained in our laboratory demonstrated that undernutrition induced marked modifications to the plasma and abomasal fluid disposition kinetics of ABZ and its metabolites in the calf (Sánchez *et al.* 1996). Higher plasma AUC and delayed elimination of ABZSO and $ABZSO_2$ were observed after intraruminal administration of ABZ in feed restricted compared with the control calf. These changes observed in the disposition kinetics may reflect an impairment on the hepatic metabolism and clearance of ABZ in animals under a poor nutritional condition. Based on these previous findings, the aim of the current research was to characterize the enantioselective liver microsomal sulphoxidation of ABZ and to investigate the influence of nutritional condition on this biotransformation process in cattle.

Materials and methods

Animals

Six male Holstein calves (120–150 kg) in a parasite-free condition were used in this trial as a source of liver material. Animals were divided in two groups. In Group A the calves were in an 'optimal' nutritional status (control group) and were fed *ad libitum* with lucerne hay plus a grain-based concentrate diet. Animals in Group B were subjected to a dietary restriction of 30 days, where only 60 % of the animal's maintenance requirements was covered, to reach a poor nutritional condition (restricted group). This lower feed intake level simulated a period of inadequate nutrition. Both experimental groups received water *ad libitum*. The food intake required to cover only the 60 % of energy requirements was estimated following established nutritional criteria for beef cattle (NRC 1984).

Biochemical determinations

Serum concentrations of free fatty acid (FFA), β-hydroxybutyrate (β-OHB) and total proteins were measured in animals from both experimental groups. These biochemical 'markers' were determined to correlate potential changes on ABZ liver microsomal sulphoxidation with metabolic disorders taking place in the animals subjected to a period of undernutrition. FFA serum concentrations were determined according to a technique adapted from that of Falholt *et al.* (1973), using palmitic acid as a standard to prepare calibration curves. β-OHB concentrations were measured using test kits from Sigma Chemical Co. (St Louis, MO, USA) following a procedure reported by Gibbard and Watkins (1968). Serum protein concentrations were determined using a routine technique (Lowry *et al.* 1951). FFA serum concentrations were measured in calves of both experimental groups at 70, 60 and 45 days before the beginning of the experimental work. Values obtained during this period were used as controls (optimal nutritional condition). At day 0, calves in the control group ($n = 3$) were sacrificed to obtain liver microsomes; animals in Group B ($n = 3$) were subjected to a 30-day restriction period (restricted group). FFA serum concentrations were measured at day 0 (control and restricted group) and at 7, 15 and 30 days after starting the period of undernutrition. β-OHB was measured in serum and in liver homogenates at the time of sacrifice in the control and restricted calf. Serum proteins were measured in both experimental groups at the sacrifice of the animals. Additionally, liver cytosolic glucose 6-phosphate dehydrogenase (G6PD) activity was determined using a kit from Sigma (Lohr and Waller 1974). The activity of this enzyme is useful in the evaluation of the NADPH generation in the pentose phosphate pathway (Madsen 1983) and its measurement was considered important to indicate nutritionally induced metabolic alterations in the feed-restricted calf.

Preparation of microsomes

Animals were sacrificed by captive bolt and exanguinated immediately. The abdomen was opened and the liver removed and rinsed with ice-cold phosphate buffer (0.01 M, pH 7.3). Liver samples were transported to the laboratory kept in the same phosphate buffer at 4 °C. All subsequent operations were performed between 0 and 4 °C. For each calf, liver samples (10–15 g) were cut into small pieces with scissors and washed several times with the phosphate buffer to remove haemoglobin. Samples were homogenized in a Ultra-Turrax homogenizer (IKA Works, Inc., Wilmington, DE, USA), centrifuged at 10000 *g* for 20 min and the resulting supernatant at 100000 *g* for 60 min. The pellet (microsomal preparation) was suspended in 0.01 M phosphate buffer and stored at −70 °C until used for incubation assays. An aliquot of the microsomal preparation was used to determine protein content using bovine serum albumin as a standard (Lowry *et al.* 1951). Preparation of liver microsomes was performed according to the techniques described elsewhere (Galtier *et al.* 1986, Souhaili El-Amri *et al.* 1987).

Enzyme assays

Sulphoxidation of ABZ was assessed by the amount of either (total), (+) or (−) ABZSO formed in the presence of the reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH) (Sigma). A typical reaction mixture contained, in a final volume of 1 ml, 1 μmol NADPH, 0.01 M phosphate buffer (pH 7.3), 100 μl tissue preparation (1 mg microsomal protein) and variable ABZ (99% pure) concentrations (0.5–40 μM). Incubations were carried out in glass vials in an oscillating water bath at 37 °C for 30 min under aerobic conditions. Incubations either without microsomes or without NADPH were carried out under the same conditions and used as blank controls. Incubations were stopped by the addition of 1 ml acetonitrile and incubated samples were frozen at −20 °C until analysis. The reaction was linear up to 30 min. All incubations were performed by duplicate and repeated several times.

Enantioselective sulphoxidation was assessed by the amount of (+) and (−) ABZSO formed after ABZ incubation with liver microsomes. Incubations were carried out with ABZ concentrations ranging from 5 to 40 μM. The comparative ABZ sulphoxidation in liver microsomal fractions obtained from the control and restricted calf was characterized by the production of (total), (+) and (−) ABZSO. Two enzyme assays were conducted for this purpose: (total) ABZSO formation was evaluated with ABZ concentrations ranging from 2 to 20 μM; in a second assay, sulphoxidation was characterized by the formation of either (total), (+) or (−) ABZSO using ABZ concentrations in the range 5–40 μM.

Sample clean-up/extraction

The sample clean-up/extraction process was performed according to a technique adapted from that described by Hennessy *et al.* (1985) and modified by Lanusse and Prichard (1990). Oxibendazole (OBZ) (0.5 μg) as internal standard was added to an aliquot (0.5 ml) of liver microsomes. Incubated and fortified samples were mixed with 0.5 ml acetonitrile, vortexed for 15 s and centrifuged at 10000 *g* for 5 min. The supernatant was mixed with 5 ml deionized water and injected into C_{18} cartridges (Lichrolut®, Merck KGaA, Darmstadt, Germany) preconditioned with methanol and deionized water. After elution with deionized water (1 ml) and methanol (2 ml), samples were evaporated to dryness under a stream of N_2. The dry residue was redissolved in 300 μl HPLC mobile phase.

Table 1. Mean (±SD) recovery percentages and linearity test of the calibration curves for albendazole (ABZ), albendazole sulphoxide (ABZSO) and albendazole sulphone ($ABZSO_2$) obtained after HPLC analysis of standards prepared in boiled (inactivated) liver microsomes.

	Recovery (%)			Deviation from linearity test*	
Analyte	Mean (±SD)	CV (%)	r	F	p
ABZ	85.1±3.4	3.99	0.998	0.08	0.970
ABZSO	83.2±4.6	5.61	0.999	1.75	0.231
$ABZSO_2$	84.2±2.1	2.49	0.999	2.57	0.119

CV, coefficient of variation.

r, Correlation coefficient.

Recovery results are derived from five determinations of each of the following concentrations: 0.25, 0.5, 1, 2, 5 and 10 $\mu g.ml^{-1}$. *, Results of this test confirm the linearity of the calibration lines for each analyte.

$p > 0.05$ indicate that the departure from linearity is not significant.

HPLC analysis

Experimental and fortified samples were analysed for ABZ, ABZSO and $ABZSO_2$ by HPLC. Fifty microlitres was injected into a Shimadzu 10 A HPLC system (Shimadzu Corp., Kyoto, Japan) fitted with a Selectosil C_{18} (5 μm, 250 × 4.60 mm) reverse-phase column (Phenomenex, CA, USA) and UV detector (Shimadzu, SPD-10A UV detector) reading at 292 nm. The rest of the HPLC analytical conditions were as previously reported (Lanusse and Prichard 1990). The analytes were identified with the retentions of 97–99% pure reference standards. Under these chromatographic conditions the mean retentions were 6.10 min (ABZSO), 7.51 min ($ABZSO_2$), 10.57 min (OBZ) and 12.87 min (ABZ).

After reverse-phase HPLC analysis, 200 μl of each extracted sample were evaporated to dryness under a N_2 stream and redissolved with 150 μl chiral mobile phase (1% 2-propanol in 0.008 M Na_2HPO_4 buffer, pH 6.9). Of each sample 50 μl were injected into the same HPLC system fitted with a chiral stationary phase column (5 μm, 100 × 4.0 mm) (Chiral-AGP column, ChromTech, Hägersten, Sweden). This chiral chromatographic method was adapted from the methodology described by Delatour *et al.* (1990). ABZSO enantiomers were identified after chromatographic analysis of a 99% pure racemic standard of this molecule. The mean retentions of both enantiomers were 3.35 min for (−) ABZSO and 6.35 min for (+) ABZSO. The relative proportions (%) of (−) and (+) ABZSO were obtained using the integrator software (Class LC 10, Shimadzu) of the HPLC system. The solvents (Baker, Inc., Phillipsburg, NJ, USA) used during the extraction and drug chromatographic analysis were of HPLC grade.

Drug/metabolite quantification

A complete validation of the analytical procedures for extraction and quantification of ABZ, ABZSO and $ABZSO_2$ was performed before starting analysis of experimental samples from the incubation trials. Known amounts of each analyte (0.25–10 $\mu g.ml^{-1}$) were added to aliquots of boiled (inactivated) liver microsomes, extracted and analysed by HPLC (triplicate determinations) to obtain calibration curves and percentages of recovery. Linearity was used to determine the concentration–detector response relationships by injection of spiked standards in liver microsomes at different concentrations. Calibration curves were prepared using the least-squares linear regression analysis of HPLC peak area ratios of the analytes/internal standard and nominal concentrations of spiked samples. Correlation coefficients (r) ranged from 0.998 to 0.999. Drug/metabolite recoveries were established by comparison of the detector responses (peak areas) obtained for microsomal fortified samples and those obtained from the injection of standards prepared in mobile phase. The mean (±SD) recovery percentages, correlation coefficients and the results of the lack-of-fit test, which confirms the linearity of the regression lines for each analyte, are summarized in table 1. The interassay precision of the analytical method was estimated by processing replicates ($n = 5$) of fortified microsomal standards of each molecule at 0.5 and 2 $\mu g.ml^{-1}$ at different days; the coefficient of variation (CV) (method's precision) for the different analytes ranged from 1.7 to 16.9%. The quantification limit for ABZ, ABZSO and $ABZSO_2$, reading at 292 nm, was 0.1 $\mu g.ml^{-1}$; this was the lowest concentration detected with a CV < 20%.

Data analysis and statistics

The reported data are expressed as mean±SD. Biochemical determinations in control and feed-restricted animals were statistically compared by an unpaired Student t-test (INSTAT 3.00, Graph Pad Software, 1997). Maximal velocity of ABZ sulphoxidation (V_{max}) and the concentration of ABZ giving half maximal velocity (K_m) were obtained after analysis of linear double reciprocal (Lineweaver–Burk)

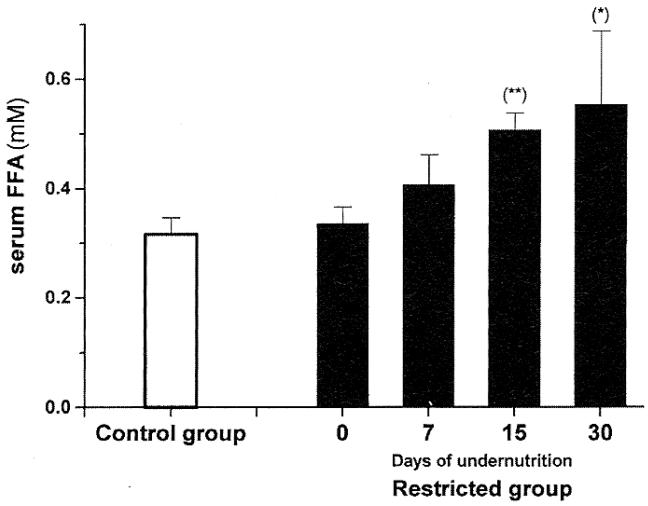

Figure 1. Mean serum FFA concentrations measured in the control and the feed-restricted calf. FFA values obtained after 15 days (**$p < 0.01$) and 30 days (*$p < 0.05$) of undernutrition were statistically different from those obtained in the control group and before the period of feed restriction. Values are the mean ($\pm$SD) of at least three determinations.

plots. The reciprocal velocity of ABZ sulphoxidation was calculated as a function of the reciprocal of ABZ concentration in these graphical plots using the INSTAT 3.00. Statistical comparison of V_{max} and K_m was performed using the non-parametric Mann–Whitney U-test. $p < 0.05$ was considered as statistically significant.

Results

Biochemical determinations

FFA serum concentrations were significantly higher in feed-restricted (poor nutritional condition) compared with control animals in an optimal nutritional condition. A gradual increase in the serum concentration of this biochemical marker was observed during the period of feed restriction. Comparative FFA serum concentrations in calves of both experimental groups are shown in figure 1. Table 2 shows the mean serum and liver β-OHB concentrations and serum protein concentrations measured in fed *ad libitum* (control group) and restricted calf. Liver cytosolic G6PD activity was significantly greater ($p < 0.05$) in the control group (13.5 ± 1.73 U.g^{-1} protein) compared with the feed-restricted group (9.08 ± 1.08 U.g^{-1} protein).

Albendazole microsomal sulphoxidation

ABZ was metabolized to its pharmacologically active sulphoxide metabolite by liver microsomes obtained from both the control (optimal nutritional condition) and feed-restricted (poor nutritional condition) calf. Under these experimental conditions, only trace amounts of $ABZSO_2$ were recovered in some microsomal incubation mixtures. The maximal rate of ABZ sulphoxidation (0.41 nmol ABZSO formed per min.mg^{-1} microsomal protein) was obtained using 1 μmol NADPH in the incubation mixture. No measurable ABZ sulphoxidation occurred in blank incubations either without microsomes or in the absence of NADPH (data not shown).

The sulphoxidation of ABZ (concentration range 5–40 μM) by liver microsomes

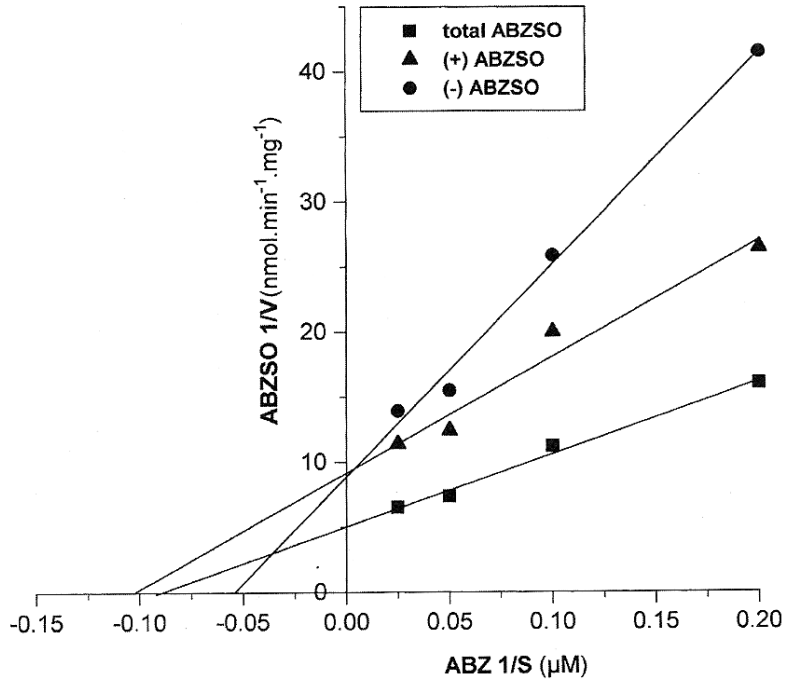

Figure 2. Lineweaver–Burk double reciprocal plot of (total), (+) and (−) ABZSO production by liver microsomes from the control (fed *ad libitum*) calf. Incubations were performed with albendazole concentrations ranging of 5–40 μM. Values are the mean (±SD) of at least six determinations.

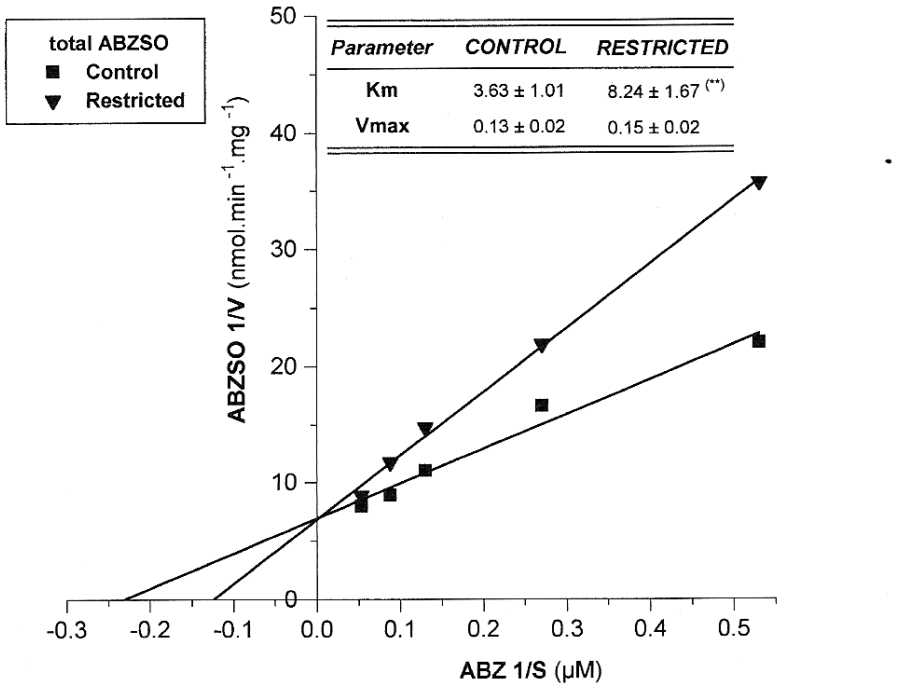

Figure 3. Lineweaver–Burk double reciprocal plot and Michaelis–Menten constants for ABZ sulphoxidase activity in liver microsomes from the control (fed *ad libitum*) and the feed-restricted calf. Incubations were performed with albendazole concentrations in the range 2–20 μM. V_{max} (nmol. $min^{-1}.mg^{-1}$), maximal velocity of ABZ sulphoxidation; K_m (μM), substrate concentration giving half-maximal velocity. **Value statistically different from that obtained in the control group at $p < 0.01$. Values are the mean (±SD) of at least six determinations.

Table 2. Mean (±SD) concentrations of β-hydroxybutyrate in serum and liver homogenates (obtained during preparation of microsomes) and serum proteins obtained in control and restricted calf.

Experimental group	β-hydroxybutyrate (mM)		Serum protein ($g.dl^{-1}$)
	Serum	Liver homogenates	
Control	0.20±0.05	0.34±0.07	6.34±0.14
Restricted	0.50±0.10*	3.03±0.98**	5.41±0.18**

Data are mean±SD of at least 3 determinations.
* Statistically different from control, $p < 0.05$.
** Statistically different from control, $p < 0.01$.
$g.dl^{-1}$; group per deciliter.

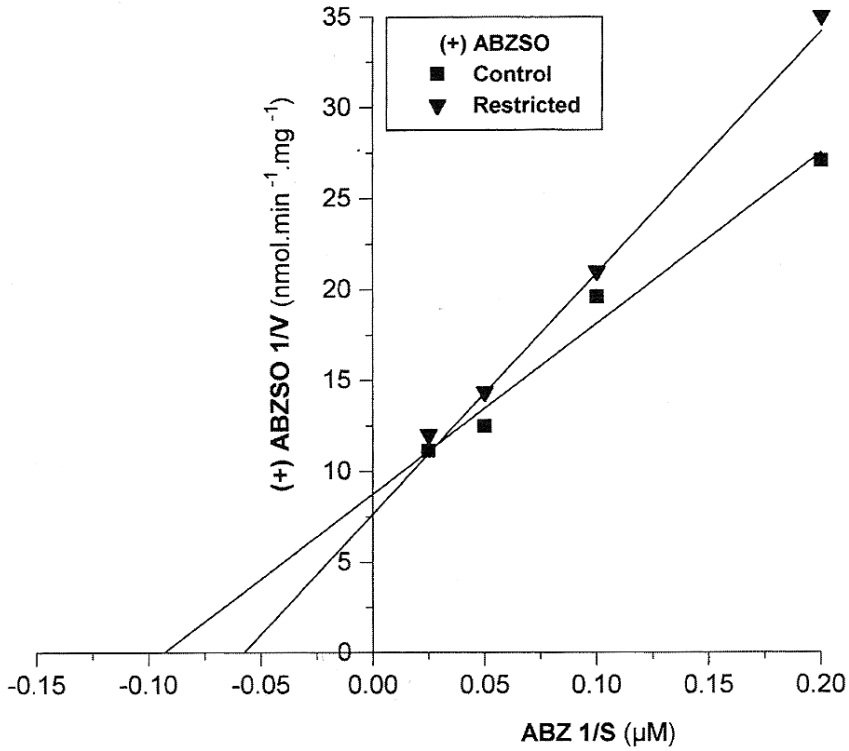

Figure 4. Lineweaver–Burk double reciprocal plot of (+) ABZSO production by liver microsomes from the control (fed *ad libitum*) and feed-restricted calf. Incubations were performed with albendazole concentrations in the range 5–40 µM. Values are the mean (±SD) of at least six determinations.

(control calf) displayed a $V_{max} = 0.27$ nmol ABZSO formed per $min.mg^{-1}$ microsomal protein and a $K_m = 15.10$ µM. Under the same ABZ concentration range, V_{max} for (+) and (−) ABZSO formation was 0.11 and 0.16 $nmol.min^{-1}.mg^{-1}$ respectively. The linear double reciprocal (Lineweaver–Burk) plots characterizing the reactions, where (total), (+) and (−) ABZSO are formed, are shown in figure 2.

Changes on the pattern of ABZ microsomal sulphoxidation were observed in the feed-restricted (subjected to a period of undernutrition) compared with the control calf. The comparative Lineweaver–Burk double reciprocal plots and the kinetic data (K_m, V_{max}) for the production of (total) ABZSO in the control compared with the feed-restricted animal are presented in figure 3. A higher K_m for the production of the (+) ABZSO enantiomer was observed after ABZ incubation with liver microsomes obtained from the feed-restricted compared with those of the control

Table 3. Michaelis–Menten constants (K_m and V_{max} values) for albendazole sulphoxidation by liver microsomes obtained from control and the feed-restricted calf.

	Control			Restricted		
Metabolite	K_m (μM)	V_{max} (nmol.min^{-1}.mg^{-1})	Cl_{int} (ml.min^{-1}.mg^{-1})	K_m (μM)	V_{max} (nmol.min^{-1}.mg^{-1})	Cl_{int} (ml.min^{-1}.mg^{-1})
(total) ABZSO	15.1±3.9	0.27±0.08	0.018±0.001	21.4±1.0*	0.28±0.02	0.013±0.001***
(+) ABZSO	9.4±3.7	0.11±0.04	0.012±0.001	18.2±2.2**	0.13±0.04	0.007±0.001***
(−) ABZSO	26.7±10.7	0.16±0.06	0.006±0.001	26.5±3.8	0.16±0.01	0.006±0.001

Data are mean±SD of at least 6 determinations.

V_{max}: maximal velocity of ABZ sulphoxidation; K_m, substrate concentration giving half-maximal velocity; Cl_{int} (*in vitro* intrinsic clearance), mean V_{max}/K_m ratio.

ABZ concentrations are between 5 and 40 μM.

* Statistically different from control, $p < 0.05$.

** Statistically different from control, $p < 0.01$.

*** Statistically different from control, $p < 0.001$.

calf (table 3, figure 4). The relative proportions of both ABZSO enantiomers, the enantiomeric excess and the enantiomeric ratio after ABZ incubation with liver microsomes obtained from both experimental groups are summarized in table 4.

Discussion

A complete characterization of the liver microsomal sulphoxidation of ABZ in cattle was carried out. The sulphoxidation of ABZ displayed a $V_{max} = 0.27$ nmol ABZSO formed per $min.mg^{-1}$ microsomal protein. This was $\sim 64\%$ lower than that obtained after ABZ incubation with sheep liver microsomes (Galtier *et al.* 1986). This finding is consistent with a 57% lower ABZ sulphoxidase activity reported for cattle than for sheep liver microsomes (Lanusse 1991). Thus, the evaluation of ABZ metabolism in the current trial confirms the lower oxidative capacity to transform ABZ in ABZSO in cattle liver microsomes compared with those of sheep.

The different Lineweaver–Burk linear relationships and K_m observed for the production of each ABZSO enantiomer might correspond with the involvement of two different enantioselective enzyme systems. The relative contributions of the cytochrome P450 and flavin monooxigenase (FMO) systems to the sulphoxidation of ABZ have been demonstrated in sheep (Galtier *et al.* 1986), pig (Souhaili El Amri *et al.* 1987), cattle (Lanusse *et al.* 1993) and rat (Moroni *et al.* 1995). Several lines of evidence support these findings, namely (1) the presence of P450 inhibitors such as *n*-octylamine and clotrimazole inhibits ABZ sulphoxidation by liver microsomes; (2) the use of an antibody to NADPH cytochrome *c* reductase also inhibits P450 isoenzymes involved in the sulphoxidation of ABZ; (3) thermal pretreatment, the use of methimazole (a FMO competitive inhibitor) and an IgG anti-FMO decreases the sulphoxidation rate of ABZ by liver microsomes from different species, and (4) the production of ABZSO from ABZ has been demonstrated using purified cytochrome P450 and FMO preparations. The enantioselectivity of the sulphoxidation of ABZ has been investigated in rat liver microsomes (Moroni *et al.* 1995). According to these *in vitro* studies the FMO system produces predominantly (+) ABZSO, whereas the P450 isoenzymes CYP2C6 and CYP2A1 are mainly involved in the production of (−) ABZSO. The enantiomeric ratio (+)/(−) ABZSO in the plasma of heifers treated with ABZ was 80/20 (Delatour *et al.* 1991a) and this level was attributed to the relative contribution of the flavin-containing and cytochrome P450-dependent oxygenases to ABZ enantioselective sulphoxidation. The higher proportion of the (+) ABZSO enantiomer measured after ABZ incubation with cattle liver microsomes in the current study agrees with those findings and may also confirm the involvement of both FMO and P450 systems in the production of (+) and (−) ABZSO respectively.

Serum concentrations of FFA and β-OHB have been shown to be good indicators of nutritional condition in ruminants (Bouchat *et al.* 1981, Barnouin *et al.* 1986); plasma β-OHB represents $> 80\%$ of the total ketone bodies in cattle. Determination of β-OHB serum concentrations is useful in the diagnosis of subclinical ketosis and fatty liver disease. The enhancement of serum FFA concentrations may be a consequence of reduced glucose availability and may be used to indicate the adequacy of feed energy supply (Riis 1983). Thus, the poor nutritional status was biochemically demonstrated by the higher serum FFA (figure 1) and β-OHB (table 2) concentrations observed in the dietary-restricted compared with the control calf. FFA concentrations also reflect changes in metabolic turnover

Table 4. Comparative chiral sulphoxidation of albendazole in liver microsomes obtained from the control (fed *ad libitum*) and the feed-restricted calf.

ABZ concentration (μM)	Control group (+) ABZSO (%)	(−) ABZSO (%)	EE	Ratio (±)[a]	Restricted group (+) AVZSO (%)	(−) ABZSO (%)	EE	Ratio (±)
0.5	76.3	23.7	52.6	3.23	72.7	27.3	45.4	2.67*
1	74.6	25.4	49.2	2.96	69.5	30.5	39.0	2.28*
5	59.4	40.6	18.8	1.47	53.9	46.1	7.8	1.18*
10	54.1	45.1	9.0	1.18	52.3	47.7	4.6	1.09
20	50.1	49.9	0.2	1.00	50.8	49.2	1.6	1.03
40	50.0	50.0	0.0	1.00	50.6	49.4	1.2	1.03

Data are mean ± SD of at least six incubations.

EE, enantiomeric excess.

[a] Average ratio between the amount (nmol mg^{-1} microsomal protein) of (+) and (−) ABZSO found in different assays.

* Ratio (±) statistically different from control, $p < 0.05$.

in the adipose tissue. During starvation, triacylglycerols are hydrolysed and FFA leave the adipocyte, diffuse to the bloodstream where they are transported to the liver bound to albumin (Salway 1994). FFA are precursors of ketone bodies in liver cells; up to 38 % of FFA taken up is used for ketone body production in the liver of the fasted rat (Madsen 1983). Thus, serum FFA concentrations may reflect β-OHB production in the liver; the ketone body diffuses freely across the mitochondrial membrane and even out of the hepatocyte, according to their concentration gradient. The higher β-OHB levels found in liver homogenates (table 2) in the calf subjected to undernutrition suggest an overproduction and its further diffusion to plasma. Liver synthesis of export proteins (e.g. plasma proteins) also seems to adapt to nutritional conditions; protein depletion and fasting limit protein synthesis in the liver (Madsen 1983). Thus, a reduced liver synthesis/enhanced catabolism may have accounted for the lower serum protein concentration observed in the animals subjected to a period of undernutrition in the current trial.

FFA, β-OHB and protein concentrations were determined to correlate potential changes on ABZ sulphoxidation with metabolic changes taking place in the animals subjected to a period of dietary restriction. This mild and sustained period of undernutrition was intended to simulate a period of inadequate nutrition to which parasitized cattle reared under field conditions may be subjected. The pattern of liver ABZ sulphoxidation was affected by the nutritional condition of the animals. No modifications on the V_{max} were observed between the control and feed-restricted calf. The higher K_m obtained for the production of (total) ABZSO in liver microsomes from animals in a poor nutritional condition suggests a reduction in the affinity of microsomal mixed-function oxidases responsible for ABZ oxidation. Furthermore, the changes observed in ABZ sulphoxidation in liver microsomes obtained from feed-restricted animals may be a consequence of the altered FMO-mediated formation of the (+) ABZSO enantiomer. The higher (+)/(−) ABZSO ratio (table 4) measured in the control calf may confirm these findings; this ratio is a good indicator of the chiral sulphoxidation of ABZ based on the relative concentrations of both enantiomers. These results seem to indicate that nutritionally induced biochemical changes account for the impairment in the FMO-mediated ABZ sulphoxidation. Delayed ABZSO and sulphone elimination (longer $T_{\frac{1}{2}el}$ and MRT) with extended detection in plasma, was the most relevant kinetic alteration

observed after ABZ administration in calves in a poor nutritional condition compared with control animals fed *ad libitum* (Sánchez *et al.* 1996). The lower Cl_{int} (table 3) obtained for the (total) and (+) ABZSO production by liver microsomes obtained from feed-restricted animals is in agreement with the pharmacokinetic alterations observed in calves under a poor nutritional condition.

At the moment one can only speculate on the molecular mechanisms mediating this metabolic interference. NADPH provides the 'reducing power' in the biosynthesis of fatty acids, cholesterol and in the production of glutathione (Salway 1994). In ruminants, ~ 75 % of NADPH is generated in the cytosol through glucose oxidation in the pentose phosphate pathway by the activity of G6PD and 6-phospho-gluconate dehydrogenase. Additionally, the activity of cytosolic isocitrate dehydrogenase produces ~ 25 % of NADPH requirements in ruminant tissues (Leat 1983), and its activity has been shown to be lower in starved animals as a consequence of depletioned levels of citrate and isocitrate. Thus, the pentose phosphate pathway may be the major source of NADPH production in calves subjected to undernutrition. Detoxifying metabolic pathways also use NADPH as a reducing equivalent; it is a substrate for glutathione reductase, being relevant in the production of the nucleophilic tripeptide glutathione (GSH), which can participate in the conjugation of electrophilic compounds including reactive oxygen species (Alvares and Pratt 1990, Salway 1994). Lipid peroxidation is an oxidative process involved in degradation of membrane lipids; peroxidative damage of membrane lipids can be assessed by determination of aldehidic products of lipid peroxidation such as malondialdehide (MDA) and 4-hydroxinonenal (HNE) (Castro and Castro 1990). It has been shown that both MDA and HNE modify the liver microsomal membrane structure and inactivate some cytochrome P450 isoforms (Buko *et al.* 1997). The reduced activity of G6PD measured in the current trial may have accounted for a decreased production and availability of NADPH in the hepatocytes of calves with a poor nutritional condition causing a negative impact in the metabolism of reactive oxygen species involved in lipid peroxidation. Interestingly, higher MDA levels, lower GSH concentrations and lower superoxide dismutase activity were observed in the liver of calves subjected to undernutrition compared with control calves fed *ad libitum* (Sansinanea *et al.* 1998). These findings suggest that hepatic antioxidant systems are altered in feed-restricted animals. The overproduction of MDA under those conditions may have accounted for an impairment in the liver drug-metabolizing capability. The FMO system is located primarily in the microsomal membrane and MDA may affect its metabolic activity. Such an effect could explain the reduced affinity of the FMO system for the substrate (ABZ) observed in liver microsomes obtained from the feed-restricted calf; this was reflected by the higher K_m obtained. Furthermore, an impairment of the FMO enzymatic system induced by the poor nutritional condition of the animals is consistent with the changes observed in the production of the (+) ABZSO enantiomer, which has been shown to be formed in a FMO-mediated ABZ oxidation process.

Finally, the enantioselective biotransformation of ABZ by cattle liver microsomes and the effects of nutritional condition on the production of each enantiomer has been reported for the first time. These results from the *in vitro* characterization of the influence of nutritional condition on the pattern of liver microsomal oxidation of ABZ correlate with the changes on its *in vivo* disposition observed in calves under poor nutritional condition (Sánchez *et al.* 1996). Regardless of the identification of

the specific mechanisms affecting ABZ biotransformation, the results reported here are a further contribution to the understanding of different factors altering the kinetic and metabolic processes of anti-parasitic drugs in ruminants.

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